REMARKS

Claims 1, 9-13, 15-16 and 22-32 have been amended. New claims 34-38 have been added. Claim 33 has been canceled without prejudice or disclaimer. Claims 2-8, 14 and 17-21 were previously canceled, and claim 33 was previously withdrawn. Subsequent to the entry of the present amendment, claims 1, 9-13, 15-16 and 22-32 are pending and at issue. These amendments and additions add no new matter as the claim language is fully supported by the specification and original claims.

I. Amendment to the Claims

Claims 1, 9-13, 15-16 and 22-32 have been amended. Independent claim 1 has been amended to recite:

> A human embryoid body derived (EBD) cell characterized by:
>
> embryoid body derived (EBD) cells derived from a human embryoid body, wherein the embryoid body is derived from embryonic germ cells, which are derived from primordial germ cells, and wherein the EBD cells proliferate in culture upon enzymatic disaggregation from the embryoid body in culture conditions not having at least one of leukemia inhibiting factor or a fibroblast feeder layer or both.

Independent claim 22 has been amended to recite:

> A method of obtaining a human embryoid body derived (EBD) cell comprising:
>
> (a) culturing primordial germ cells under conditions that are suitable for formation of a solid or cystic embryoid body having a 3-dimensional morphology;
>
> (b) disaggregating the solid or cystic embryoid body under suitable enzymatic conditions to provide a constituent cell or embryoid body derived (EBD) cell; and
>
> (c) culturing the EBD cell under conditions suitable to produce a population of proliferating EBD cells.

Dependent claims 9-13, 15-16 and 23-32 have been amended to improve their form and incorporate the amendments of claims 1 and/or 22. Claims 25 and 26 have been amended to replace "hFGF" with "hFGF-2", which has support in the specification in paragraph [0183].

Claim 27 has been amended and the term "matrix" is supported in at least paragraphs [0012] and [0017] of the specification.

Also, the claims make clear that "embryoid body derived cell (EBD)" are produced from "embryoid body" which are produced from the "embryonic germ cells", which are derived from the "primordial germ cells". Definitions of these and other terms and phrases are provided in the specification, e.g., at least paragraphs [0008]-[0012]; [0030] to [0033] and Examples 3 & 4. Moreover, the claims are clear that the EBD cells were made my enzymatic disaggregation of the embryoid body (paragraph [0130] of the specification) and their proliferation on a surface matrix. This is in contrast to any of the cited references alone and/or combined (discussed in more detail below).

New claims 34-38 have been added and are fully supported in the specification and original claims e.g., Example 3 and paragraph [0130].

The amendments do not add new matter.

II. Rejections under 35 U.S.C. § 112, Second Paragraph

A. Claims 22-32 are rejected under 35 U.S.C. §112, second paragraph, as being allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Applicants traverse the rejection as it may apply to the pending claims.

According to the Office Action, "[c]laim 23 lacks antecedent basis in claim 22 for the term "EBD cell culture." Further, claim 23 is confusing as claim 22 states "a constituent cell" and claim 23 states "selecting a single cell." ....Claims 22 states "reduced serum." However, reduced serum is not defined in the specification nor does it have a clear meaning in the art. The only definition of "reduced serum" in the specification is 5% (page 67, line 6). Applicant needs to

point to clear definition of the term so that the metes and bounds are known (page 2 of the Office Action)".

Claim 22 has been amended as discussed above to recite "[a] method of making a human embryoid body derived cell…", and claim 23 has been amended to recite "[t]he method of claim 22 comprising selecting a single embryoid body derived cell from the embryoid body derived cells and culturing the single embryoid body derived cell to produce a clonal population of cells". Hence, claim 22 provides antecedent basis for dependent claim 23, and claim 23 further limits claim 22, as it is selecting "a single EBD cell from the EBD cells". Claim 22 has also been amended to delete the phrase "reduce serum", hence this rejection is moot with regards to the alleged indefiniteness of this phrase.

Accordingly, withdrawal of rejection of claims 22-32 under 35 U.S.C. §112, second paragraph is respectfully requested.

III. Rejections under 35 U.S.C. §102

A. Claims 1 and 9-13 are rejected under 35 U.S.C. §102(b) as being allegedly anticipated by Shamblott et al (1998) *Proc. Natl. Acad. Sci.* 95, pp. 13726-13731 (hereinafter, "Shamblott"). Applicants respectfully traverse this rejection as it applies to the pending claims.

To anticipate, a single reference must inherently or expressly teach each and every element of claimed invention. *In re Spada*, 15 USPQ2d 1655 (Fed Cir. 1990); and *Verdegaal Bros. v. Union Oil Co. of California*, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). MPEP §2131. Further, the claimed invention must be distinct from what is apparently inherent in the reference, and the reference must be enabling to place the allegedly disclosed matter in the possession of the public. *In re Fitzgerald et al.*, 619 F.2d 67, 205 USPQ 594 (CCPA 1980); and *Akzo N.V. v. U.S. Int'l Trade Comm'n*, 1 USPQ2d 1241, 1245 (Fed. Cir. 1986).

According to the Office Action, "Shamblott teaches embryoid bodies (EB's) produced from human primordial germ cells (hPGC's) (13729, col. 1, parag. 1-12). The EB's are taught by Shamblott to express markers of each embryonic germ layer: endoderm, mesoderm and ectoderm (page 13729, Table 1). In particular, Shamblott shows the EB's to express nonphosphorylated neurofilament heavy isoform, as indicated by reactivity with sm311, and to express a-fetoprotein, as indicated by reactivity with A008 (page 13728, col. 2, lines 2-4). An embryoid body is an enriched population of cells. Further, since the EB's taught by Shamblott and those disclosed in the present application are made by the same method, originating from hPGC's and have the same embryonic germ layers as indicated by marker expression, the EB cells of Shamblott would be expected by the ordinary artisan to undergo 30 or 60 population doublings. Further, the culture conditions taught by Shamblott for the culture of EB's clearly is non-permissive for human embryonic germ cells, and lacks LIF or a fibroblast feeder layer (page 13727, col. 1, parag. 2, lines 1-4). The cells of Shamblott inherently can be infected by a retrovirus or a lentivirus. Thus, Shamblott clearly anticipates the claimed invention (pages 4-5 of the Office Action)".

Claim 1 has been amended to recite:

> A human embryoid body derived (EBD) cell characterized by:
> human embryoid body derived (EBD) cells derived from a human embryoid body, wherein the embryoid body is derived from embryonic germ cells, which are further derived from primordial germ cells, and wherein the EBD cells proliferate in culture upon enzymatic disaggregation from the embryoid body.

Claim 1 is fully supported by the application as filed, and is also distinguished from Shamblott as follows:

First, paragraphs [0019], [0030] and [0041] of the specification describe that embryoid body derived cell(s) (EBDs) are derived from embryoid bodies (EBs), which

are derived from embryonic germ cells, which are then derived from primordial germ cells (PGCs):

> [0019] We have isolated cells *from* human embryoid bodies (EBs), termed "embryoid body-derived (EBD) cells," that are capable of long-term and robust proliferation in culture. [emphasis added]
>
> [0030] The term "embryoid bodies" or "EBs" refers to collections of cells formed from the aggregation or clustering of cultured embryonic germ (EG) cells in culture, as described herein. EBs have a three dimensional morphology, e.g., they can be a solid or a cystic embryoid body.
>
> [0041] Human embryoid bodies (EBs) form spontaneously in human PGC-derived stem cell cultures (see Example 1, below) …

In contrast, Shamblott in FIGs.2-3 disclose embryoid bodies that have 3-dimensional morphology and appear as "colonies" or clumps (page 13727, col.2). Shamblott also discloses that "PGCs gave rise to *large tightly compacted multicellular colonies* resembling early passage mouse ES or EG cell colonies (emphasis added; page 13729, col. 1, Results, first paragraph, lines 10-11)". Thus, Shamblott does not disclose the claimed embryoid body derived cell(s).

The claimed invention and that of Shamblott are also distinguished because the cells constituting the embryoid cells (EBs) of Shamblott do not and *cannot* "proliferate in culture unless they are enzymatically disaggregated from the embryoid body" as in the claimed invention. Shamblott does not disclose "disaggregation" of cells from the embryoid bodies.

This characteristic of the claimed embryoid body derived cell(s) is supported in the application as filed, for example, paragraph [0130] of Example 3 describes that:

> … EBs are physically removed from the stem cell culture medium where they are formed, and placed in a calcium and magnesium-free phosphate-buffered saline (PBS). The EBs are then sorted into categories by gross morphology, e.g., cystic or solid. *After sorting, the EBs are transferred to a mixture of one mg/ml collagenase and dispase enzyme (Boehringer Mannheim), and incubated for 30*

> *minutes to three hours at 37°C; during this time they are manually agitated or triturated every about 10 to 30 minutes.* Other *dissociation treatments can be used*, e.g., the individual or combined use of several different types of collagenase, dispase I, dispase II, hyaluronidase, papain, proteinase K, neuraminidase and/or trypsin. Each treatment requires optimization of incubation length and effectiveness; cell viability can be monitored visually or by trypan blue exclusion followed by microscopic examination of a small aliquot of the disaggregation reaction. One collagenase/dispase disaggregation protocol calls for incubation for about 30 minutes at 37°C; this results in between about 10% and 95% of the EB constituent cells disaggregated into single cells. Large clumps of cell may remain intact. [emphasis added]

Thus, embryoid body derived cell(s) proliferate in a culture *after* they have been enzymatically disaggregated from the embryoid bodies, e.g., in the presence of collagenase/dispase. In contrast, Shamblott does *not* disclose "disaggregation" of the embryoid bodies (EBs). Shamblott uses the term "disaggregation" with reference to disaggregating "gonadal ridges and mesenteries of 5- to 9-week old post-fertilization human embryos" (page 13726, col. 2, Materials and Methods, lines 2-7). In fact, Shamblott admits that "disaggregating" the embryoid bodies is difficult, for example:

> Unlike mouse pluripotent stem cells (ES and EG), these human cells were more resistant to disaggregation by trypsin/EDTA-based reagents. [page 123729, col. 1, Results, second paragraph, last sentence]

> Maintaining high colony density and derivation of clonal cell lines is complicated by the difficulties associated with disaggregation of colonies to single cells.... These interactions are notably more resistant to trypsin than mouse ES and EG colonies. [page 13729, col. 2, Discussion, third paragraph]

> Although properties of the cultures described in this paper are consistent with those of pluritpotent stem cells, the cultures have a lower plating efficiency than most mouse EG and ES cell cultures, which may reflect difficulties associated with the complete cell disaggregation. [page 13730, col. 1, first paragraph]

Lastly, as recited in new independent claim 38, the claimed EBD cells are also distinguished from embryonic germ cells (EG cells) because they can expand *directly* on a

surface or matrix, whereas EG cells require at least a fibroblast feeder layer. This is fully supported by the specification on paragraph [0060], for example:

> In culturing EG cells, it is believed that the use of feeder cells, or an extracellular matrix derived from feeder cells, provides one or more substances necessary to promote the growth of EG cells and/or prevents or inhibits the rate of differentiation of such cells.... *Such feeder cells are not needed for proliferation of EBD cells in culture...*

Thus, based on the foregoing, Shamblott cannot anticipate the claimed invention because Shamblott does *not* teach *each and every element* of claim 1 and/or claim 38.

Also, the Office Action by alleges that Shamblott on page 13727, col. 1, parag. 2, lines 1-4, discloses cultures lacking "LIF or a fibroblast feeder layer" (page 4 of the Office Action, and as quoted above). Yet, it is submitted, that the lack of LIF in this instance disclosed by Shamblott is not for proliferating of the embryoid bodies (EBs), but for "embedding" the embryoid bodies (EBs) for immunohistochemical analysis. Shamblott discloses that the embryoid bodies (EBs), not the EBD cells, are grown "in the presence" of STO fibroblast feeder layer and LIF (Abstract; page 13727, col. 1, lines 1 and 8-9; and page 13729, page 13729, col. 1, Results, first paragraph, lines 8-9). Moreover, Shamblott does not disclose EBD cell(s) as claimed, therefore Shamblott cannot disclose EBD cells proliferating in a culture conditions which are nonpermissive for EG cells (claim 11), or lack LIF, a fibroblast feeder layer or both (claim 12).

Lastly, since Shamblott does not disclose *each and every element* of claim 1, Shamblott cannot disclose that the embryoid body derived cell(s) undergo 30 to 60 population doublings as in claim 9. Shamblott discloses that the embryoid bodies (EBs) "can be continuously maintained for more than 20 passages (page 13729, col. 2, Discussion, 3rd paragraph, first sentence)". However, near the end of the Discussion, Shamblott admits that the cells have only been "passaged 20-25 times" (page 13730, col. 1, 1st paragraph, last sentence). Hence, Shamblott does not anticipate the claimed invention because Shamblott does not disclose *each and every element* of the claimed invention.

Accordingly, withdrawal of rejection of claims 1 and 9-13 under 35 U.S.C. §102, is respectfully requested.

B. Claims 22, 27 and 30-32 are rejected under 35 U.S.C. §102(b) as being allegedly anticipated by Shamblott. Applicants respectfully traverse this rejection as it applies to the pending claims.

According to the Office Action, "Shamblott teaches a method of making an enriched population of cells comprising culturing hPGC's to form cystic EB's, dissociating the cystic EB's to provide a constituent cell and culturing the constituent cells in serum containing media... The EB's were dissociated from the original culture by plating them on tissue culture plates in the absence of LIF, FGF and forskolin (page 13727, col. 1, parag. 2, lines 1-4). The tissue culture plate provides a matrix. The EB's cells are cultured in a media not permissive for EG cell growth, which are growth in the presence of LIF, FGF and forskolin (page 12727, col. 1, lines 8-11). The EB cells would inherently be capable of 30 population doublings (page 5 of the Office Action).

Claim 22 has been amended and recites:

> A method of obtaining a human embryoid body derived (EBD) cell comprising:
>
> (a) culturing primordial germ cells under conditions that are suitable for formation of a solid or cystic embryoid body having a 3-dimensional morphology;
>
> (b) disaggregating the solid or cystic embryoid body under suitable enzymatic conditions to provide a constituent cell or embryoid body derived (EBD) cell; and
>
> (c) culturing the EBD cell under conditions suitable to produce a population of proliferating EBD cells.

First, the claimed invention recites various phrases, e.g., "embryoid body derived cell(s)", "embryoid body", "embryonic germ cells", and "primordial germ cells", all of which were discussed above under the rejection with regards to claim 1. In short, the instant

application and Shamblott are in agreement that primordial germ cells give rise embryonic germ cells, which spontaneously give rise embryoid bodies in cultures.

However, the claimed invention is not directed to embryoid bodies or methods of making embryoid bodies, the claimed invention is directed to an embryoid body derived (EBD) cell and methods of making an embryoid body derived (EBD) cell. The detail discussion above, of the support for claim 1 and the distinguishing elements of claim , from that disclosed in Shamblott, are also applicable to method claim 22 and the rejection therein. For example, Shamblott clearly recites and shows in FIGs. 1-3 that the embryoid bodies are "large tightly compacted and multicellular colonies resembling early passage ES and EG cell colonies" (legend in FIG.2; and page 13729, col. 1, Results paragraph, lines 8-12; and page 13729, col. 1, Results section, second paragraph, lines 1-2; and page 13729, col. 2, Discussion, 3rd paragraph, third and fourth sentence). In contrast, the "claimed invention is a method of making a human embryoid body derived cell" (claim 22) and *not* "colonies" or embryoid bodies.

Applicants also submit that the claimed invention and the corresponding publication (Exhibit A: Shamblott et al. (2001) "Human embryonic germ cell derivatives express a broad range of developmentally distinct markers and proliferate *in vitro*" *PNAS* 98(1)113-118), both filed and published in January 2001, are the results of further investigation beyond 1998. In fact, the second paragraph of the Discussion section in Exhibit A (Shamblott 2001) discloses that as of the date of the publication of the paper, there was no description of successful *in vitro* proliferation of embryoid body derived cells (page 117 of Exhibit A). There is no *in vitro* proliferation of embryoid body derived cells, because no cells of any sort were disaggregated from the embryoid bodies. Thus, it is submitted that there is no teaching in Shamblott of a "method of making a human embryoid body derived cell" as claimed, since Shamblott *only* discloses aggregated "colonies" of embryoid bodies. Hence, Shamblott does *not* disclose, "disaggregated" cells of *any* kind, let alone EBD cells.

Moreover, the conditions described by Shamblott where there is absence of LIF as alleged by the Office Action above (claims 30-31), is for the purpose of "embedding" the embryoid bodies for immunohistochemical analysis. Shamblott discloses that the embryoid bodies were embedded in "molten 1% low melting point agarose for the purpose of immunohistochemical analysis (page 13727, col. 1, second paragraph, lines 5-6)". The embryoid bodies in agarose were further embedded in paraffin and individual 6 micron sections cut and placed on slides for immunohistochemistry analysis (page 13728, col. 1). Hence, the embedding method disclosed in Shamblott was *not* for the purpose of "culturing (claim 22(a) and (c))", and "disaggregating (claim 22(b))" the embryoid body derived cells from the embryoid bodies as claimed. In fact, as discussed above, the embryoid bodies identified in Shamblott are not capable of proliferating in culture because Shamblott discloses embryoid bodies which were never disaggregated in the first place. Please see detail discussion above. Therefore, Shamblott cannot anticipate the claimed invention as Shamblott does *not* disclose *each and every element* of the claimed invention.

Accordingly, withdrawal of rejection of claims 22, 27 and 30-32 under 35 U.S.C. §102, is respectfully requested.

IV. Rejections under 35 U.S.C. §103

A. Claims 1, 15 and 16 are rejected under 35 U.S.C. §102(b) as allegedly anticipated by or, in the alternative, under 35 U.S.C. §103(a) as obvious over Shamblott. Applicants respectfully traverse the rejection as it applies to the pending claims.

To establish a *prima facie* case of obviousness, three basic criteria must be met: 1) a suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; 2) a reasonable expectation of success; and 3) the references must teach or suggest all the claim limitations. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991); MPEP § 2143.

Additionally, it is impermissible to apply the benefit of hindsight in order to arrive at a verdict of obviousness. See, e.g., *Panduit Corp. v. Dennison Manufacturing Co.*, 227 USPQ 337, 343 (Fed. Cir. 1985), *vacated & remanded, Dennison Mfg. Co. v. Panduit Corp.*, 475 U.S. 809, L. Ed. 2d 817, 106 S. Ct. 1578, 229 USPQ 478 (1986), *on remand*, 810 F2d 1561, 1 USPQ2d 1593 (Fed. Cir. 1987), *cert. denied*, 107 S. Ct. 2187, 95 L. Ed. 2d 843 (1987), quoting *W.L. Gore Assocs., Inc. v. Garlock, Inc.*, 220 USPQ 303, 313 (Fed. Cir. 1983):

> It is difficult but necessary that the decision maker forget what he or she has been taught at trial about the claimed invention and cast the mind back to the time the invention was made (often as here many years), to occupy the mind of one skilled in the art who is presented only with the references, and who is normally guided by the then-accepted wisdom in the art.

According to the Office Action, "Shamblott teaches embryoid bodies (EB's) produced from human primordial germ cells (hPGC's) (13729, col. 1, parag. 1-12). The EB's are taught by Shamblott to express markers of each embryonic germ layer: endoderm, mesoderm and ectoderm (page 13729, Table 1). In particular, Shamblott shows the EB's to express nonphosphorylated neurofilament heavy isoform, as indicated by reactivity with sm311, and to express a-fetoprotein, as indicated by reactivity with

A008 (page 13728, col. 2, lines 2-4). An embryoid body is an enriched population of cells. The clonally derived cells of claims 14 and 15 are taught by the specification to express markers of more than one embryonic germ layer. Thus, the EB's of Shamblott, composed of cells of each germ layer, are the same cells as those of claims 14 and 15. Alternatively, the cells of claims 14 and 15 are obvious over those of Shamblott because there is no distinction between them, as both Shamblott's cells and those claimed express the same marker. The term "clonal" is a method of producing the claimed cells. If the method of producing a produce doesn't provide a novel and non-obvious property to a known product, the known product is not patentable (page 7 of the Office Action)".

Claim 1 has been amended as discussed above. Shamblott neither teaches nor suggests the claimed invention because Shamblott does not disclose disaggregation of the embryoid bodies to begin with. The embryoid bodies, or the "large tightly compacted multicellular colonies", of Shamblott maintain their 3-dimensional morphology (FIG. 1-3 of Shamblott). The embryoid body derived cell(s) of the claimed invention are *not* "colonies" nor do they have a "3-dimensional morphology" as the embryoid bodies in Shamblott. Further, one skilled in the art would understand that a "colony" is *not* a "clone" (claim 15). Clonal embryoid body derived cell(s) are described in Example 4 and transfection of these clonal embryoid body derived cell(s) are described in Example 5 of the specification. Therefore, Shamblott does not anticipate or render the claimed invention obvious, because Shamblott does not in the first instance, disclose *each and every element* of the claimed invention, or in the alternative, Shamblott neither teaches nor suggests the claimed invention.

Additionally, the results of the claimed invention were published in January 2001(Exhibit A, Shamblott (2001) as discussed above), the same month and year as the filing of the instant application; both disclosing embryoid body derived cell(s) and methods of making embryoid body derived cell(s). Exhibit A also discloses that at least between the first Shamblott publication in 1998, disclosing *only* embryoid bodies, to the date of the publication

of the Shamblott 2001 paper (Exhibit A), no successful attempts of disaggregating embryoid bodies or *in vitro* proliferation of cells from embryoid bodies were described in the literature (page 117, Discussion, second paragraph). Thus, it cannot be said that Shamblott renders the claimed invention obvious when in fact issues with regards to "disaggregating" the embryoid bodies had been unresolved up to the date of the filing of the application, or the publication of Exhibit A (Shamblott 2001).

Accordingly, withdrawal of rejection of claims 1, 15 and 16 under 35 U.S.C. §103, is respectfully requested.

B. Claims 22 and 24 are rejected under 35 U.S.C. §103(a) as being allegedly unpatentable over Shamblott in view of Vittet et al. (1996) *Blood* 88, pp. 3424-3431 (hereinafter, "Vittet"). Applicants respectfully traverse the rejection as it applies to the pending claims.

According to the Office Action, "Shamblott teaches a method of making an enriched population of cells comprising culturing hPGC's to form cystic EB's, dissociating the cystic EB's to provide a constituent cell and culturing the constituent cells in serum containing media, the constituent cells simultaneously expressing at least a first and second polypeptide or mRNA marker from at least two different cell types, wherein the cell types are selected from ectodermal cells, mesodermal cells, or endodermal cells, ... The EB's were dissociated from the original culture by plating them on tissue culture plates in the absence of LIF, FGF and forskolin (page 13727, col. 1, parag. 2, lines 1-4). Vittet teaches the growth of cells isolated from mouse EB's in the presence of human basic fibroblast growth factor to initiate differentiation into vascular structures, and observed the expression of various endothelial cell markers (page 3427, col. 1, parag. 1, lines 2-8). Thus, at the time of present invention, it would have been obvious to the ordinary artisan to culture the EB cells of Shamblott in the presence of human bFGF (FGF2) to determine the effect of the growth factor on endothelial and vascular cell development on human EB cell (pages 7-8 of the Office Action)".

Claim 22 has been amended as discussed above. Again, Shamblott does not teach, suggest or motivate one skilled in the art to make embryoid body derived cell(s) from embryoid bodies or "colonies" as claimed and described in the specification, e.g., Examples 1-4. Although Shamblott discloses that "alternative disaggregation enzymes are currently under investigation (page 13729, col. 2, Discussion, 3rd paragraph, last sentence)", it cannot be said this statement alone teaches, suggests or motivates one skilled in the art to make embryoid body derived cell(s) from embryoid bodies as claimed and described. Vittet discloses "ES-derived EBs [and]…increased development of primitive vascular-like structures *within* EBs" in a GFs cocktail (Abstract; and page 3430, col. 1, first paragraph, last sentence). The development of putative vascular-like structures is "within" (or inside) the embryoid bodies. Also, Vittet shows colonies or clumps of embryoid bodies in FIGs. 4, 6 and 7. In summary, Shamblott and/or Vittet disclose *only* embryoid bodies and not embryoid body derived cell(s) or methods of making the embryoid body derived cell(s), therefore they cannot render the claimed invention obvious.

Accordingly, withdrawal of rejection of claims 22 and 24 under 35 U.S.C. §103, is respectfully requested.

C. Claims 22, 23 and 26 are rejected under 35 U.S.C. §103(a) as being allegedly unpatentable over Shamblott. Applicants respectfully traverse the rejection as it applies to the pending claims.

According to the Office Action, "Shamblott teaches a method of making an enriched population of cells comprising culturing hPGC's to form cystic EB's, dissociating the cystic EB's to provide a constituent cell and culturing the constituent cells in serum containing media, the constituent cells simultaneously expressing at least a first and second polypeptide or mRNA marker from at least two different cell types, wherein the cell types are selected from ectodermal cells, mesodermal cells, or endodermal cells, …. At the time of the instant invention, the

formation of a clonal culture from a single cell would have been obvious to the ordinary artisan to create a cell population having the same biochemical characteristics. Further, RPMI1640 was a well-known cell culture media, and the determination of the most effective growth conditions for a cell population was within the scope of skills of the ordinary artisan at the time of the instant invention. Likewise the determination of components of a culture media to optimize growth of a cell culture was well known and within the scope of skills of the ordinary artisan at the time of the instant invention (pages 8-9 of the Office Action)".

Claim 22 has been amended to recite a method of making a human embryoid body derived cell, as discussed above. Shamblott does not disclose embryoid body derived cell(s), rather Shamblott *only* discloses embryoid bodies spontaneously arising from embryonic germ cells, arising from primordial germ cells. Claim 23, directed to selecting a "single embryoid body derived cell" and culturing it to make a "clonal population of embryoid body derived cells" cannot be said to be taught nor suggested by Shamblott since Shamblott does not disclose the claimed embryoid body derived cell(s) or methods of making the embryoid body derived cell(s) in the first place. Similarly, claim 26 is indirectly dependent on claim 22, hence the limitation of claim 26 combined with claim 22 is not rendered obvious over Shamblott, because Shamblott does not teach or suggest the claimed EBDs or methods of making the embryoid body derived cell(s).

Accordingly, withdrawal of rejection of claims 22, 23 and 26 under 35 U.S.C. §103, is respectfully requested.

D. Claims 22, 28 and 29 are rejected under 35 U.S.C. §103(a) as being allegedly unpatentable over Shamblott in view of Rohwedel et al (1996) *Cell Biol. Internat.* 20, pp. 579-587 (hereinafter, "Rohwedel"). Applicants respectfully traverse this rejection as it applies to the pending claims.

According to the Office Action, "Shamblott teaches a method of making an enriched population of cells comprising culturing hPGC's to form cystic EB's, dissociating the cystic EB's to provide a constituent cell and culturing the constituent cells in serum containing media, the constituent cells simultaneously expressing at least a first and second polypeptide or mRNA marker from at least two different cell types, wherein the cell types are selected from ectodermal cells, mesodermal cells, or endodermal cells,... Rohwedel teaches the culture of mouse EB cells on tissue culture plates coated with gelatin for morphological studies (page 580, col. 2, parag. 1, lines 14-18). It is noted that gelatin is a hydrolyzation product of collagen I. Thus at the time of the instant invention, it would have been obvious to the ordinary artisan to plate the EB cells of Shamblott on gelatin coated plates in order to study their morphology (pages 9-10 of the Office Action)".

Claim 22 has been amended to recite a method of making embryoid body derived cell(s) as discussed above. Shamblott does not disclose embryoid body derived cell(s), rather Shamblott *only* discloses embryoid bodies spontaneously arising from embryonic stem or embryonic germ cells, arising from PGCs. Rohwedel, similar to Shamblott and Vittet, does not teach or suggest embryoid body derived cell(s) derived from embryoid bodies and methods of making the embryoid body derived cell(s). Rohwedel discloses differentiation of mouse embryonic germ (EG) cell "aggregates" of line EG-1 derived from mouse PGCs (Abstract; and page 580 col. 1 &2; FIGs.1-3). Rohwedel does not teach or suggest disaggregation of the embryonic germ cell aggregates, nor does Rohwedel teach or suggest proliferation of the embryoid body derived cell(s) derived from the "aggregates". Further,

claim 29, directed to the claimed method cultured on a matrix of collagen I and human extracellular matrix, is indirectly dependent on claim 22, hence the limitation of claim 29 combined with claim 22 is not rendered obvious over Shamblott and/or Rohwedel, as neither Shamblott or Rohwedel, alone or combined, teach or suggest the claimed embryoid body derived cell(s) or methods of making embryoid body derived cell(s) in the first place. That is, Rhowedel does not cure the deficiencies of Shamblott. In summary, Shamblott and/or Rohwedel disclose *only* embryoid bodies and not embryoid body derived cell(s) or methods of making the same, therefore they cannot render the claimed invention obvious.

Accordingly, withdrawal of rejection of claims 22, 28 and 29 under 35 U.S.C. §103, is respectfully requested.

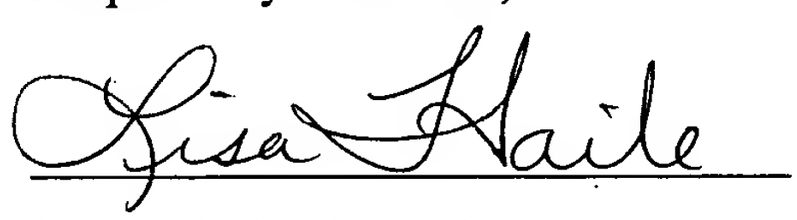
CONCLUSION

In view of the amendments and above remarks, it is submitted that the claims are in condition for allowance, and a notice to that effect is respectfully requested. The Examiner is invited to contact Applicant's undersigned representative if there are any questions relating to this application.

A check in the amount of $450.00 is enclosed as payment of the Two Month Extension of Time Fee. No other fee is deemed necessary with the filing of this paper. However if any fees are due, the Commissioner is hereby authorized to charge any fees, or make any credits, to Deposit Account No. 07-1896 referencing the above-identified attorney docket number. A copy of the Transmittal Sheet is enclosed.

Respectfully submitted,

Date: June 12, 2006



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Human embryonic germ cell derivatives express a broad range of developmentally distinct markers and proliferate extensively *in vitro*

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Human pluripotent stem cells (hPSCs) have been derived from the inner cell mass cells of blastocysts (embryonic stem cells) and primordial germ cells of the developing gonadal ridge (embryonic germ cells). Like their mouse counterparts, hPSCs can be maintained in culture in an undifferentiated state and, upon differentiation, generate a wide variety of cell types. Embryoid body (EB) formation is a requisite step in the process of *in vitro* differentiation of these stem cells and has been used to derive neurons and glia, vascular endothelium, hematopoietic cells, cardiomyocytes, and glucose-responsive insulin-producing cells from mouse PSCs. EBs generated from human embryonic germ cell cultures have also been found to contain a wide variety of cell types, including neural cells, vascular endothelium, muscle cells, and endodermal derivatives. Here, we report the isolation and culture of cells from human EBs as well as a characterization of their gene expression during growth in several different culture environments. These heterogeneous cell cultures are capable of robust and long-term [>70 population doublings (PD)] proliferation in culture, have normal karyotypes, and can be cryopreserved, clonally isolated, and stably transfected. Cell cultures and clonal lines retain a broad pattern of gene expression including simultaneous expression of markers normally associated with cells of neural, vascular/hematopoietic, muscle, and endoderm lineages. The growth and expression characteristics of these EB-derived cells suggest that they are relatively uncommitted precursor or progenitor cells. EB-derived cells may be suited to studies of human cell differentiation and may play a role in future transplantation therapies.

Mouse pluripotent stem cells (mPSCs) have been derived from the inner cell mass cells of blastocysts and from primordial germ cells colonizing the developing gonadal ridge and are referred to as embryonic stem (ES) cells and embryonic germ (EG) cells, respectively. When mPSCs differentiate *in vitro*, they form complex three-dimensional cell aggregates termed embryoid bodies (EBs). Some early developmental processes are recapitulated within the environment of an EB, resulting in a haphazard collection of precursor and more fully differentiated cells from a wide variety of lineages. Through this intermediate step, mPSCs can generate cells of the hematopoietic lineage (1, 2), cardiomyocytes (3, 4), neurons (5) and glial precursors (6), skeletal muscle (7), vascular endothelial cells (8), visceral endoderm (9, 10), and glucose-responsive insulin-producing cells (11).

When human EG cells differentiate, they also form EBs composed of endodermal, ectodermal, and mesodermal derivatives (12). Although a compelling demonstration of the potential of human EG cells, the limited growth characteristics of differentiated cells within EBs and difficulties associated with their isolation would make extensive experimental manipulation difficult and limit their use in future cellular transplantation therapies. We reasoned that precursor or progenitor cells of the observed constituent cell types must have been present at some point in EB formation, and if they could be isolated, these cells may have desirable proliferation and expression characteristics.

Here, we describe cells isolated from human EBs that are capable of long-term and robust proliferation in culture. Mixed cell EB-derived (EBD) cultures and clonally isolated EBD cell lines simultaneously express a wide array of mRNA and protein markers that are normally associated with distinct developmental lineages. The proliferation and expression characteristics of these cells suggest they may be useful in the study of human cell differentiation and as a resource for cellular transplantation therapies.

Materials and Methods

Pluripotent Stem Cell Culture, EB Formation, and Establishment of EBD Cell Cultures. Human EG cultures LV (XX), SL (XY), LU2 (XY), and SD (XX) were derived and cultured from 5-, 6-, 7-, and 11-week postfertilization primordial germ cells, respectively, as described (12). EBs were formed in the presence of leukemia inhibitory factor (1000 U/ml), basic fibroblast growth factor (2 ng/ml), forskolin (10 μM), and 15% FCS (HyClone). Approximately 10 cystic EBs from each culture were dissociated by digestion in 1 mg/ml Collagenase/Dispase (Roche Molecular Biochemicals) for 30 min to 1 h at 37°C. Cells were then spun at 1,000 rpm for 5 min and then resuspended in various growth media/matrix environments. RPMI growth media included RPMI 1640 (LTI)/15% FCS/0.1 mM nonessential amino acids/2 mM L-glutamine/100 U/ml penicillin/100 μg/ml streptomycin. EGM2MV media (Clonetics, San Diego) included 5% FCS, hydrocortisone, human basic fibroblast growth factor, human vascular epidermal growth factor, R(3)-insulin-like growth factor I, ascorbic acid, human epidermal growth factor, heparin, gentamycin, and amphotericin. Matrices were bovine collagen I (Collaborative Biomedical Products, Bedford, MA; 10 μg/cm^2), human extracellular matrix (Collaborative Biomedical Products, 5 μg/cm^2), and tissue culture plastic. Cells were cultured at 37°C, 5% CO_2, 95% humidity and routinely passaged 1:10 to 1:40 by using 0.025% Trypsin, 0.01% EDTA (Clonetics) for 5 min at 37°C. Low serum cultures were treated with trypsin inhibitor (Clonetics) and then spun down and resuspended in growth media. Cell were cryopreserved in the presence of 50% FCS, 10% DMSO in a controlled rate freezing vessel, and stored in liquid nitrogen. Cells prepared for cytogenetic analysis were

Abbreviations: PD, population doublings; ES, embryonic stem; EG, embryonic germ; EBD, embryoid body-derived; PSC, pluripotent stem cell; EB, embryoid body; EGFP, enhanced green fluorescence protein; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase.

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incubated in growth media with 0.1 μg/ml Colcemid/2.5 μg/ml ethidium bromide for 3 h, trypsinized, resuspended in 0.075 M KCl, incubated for 35 min at 37°C, and then fixed in 3:1 methanol/acetic acid. Cell proliferation assays were carried out by plating 1×10^4 cells of EBD culture SD into 35-mm dishes containing the growth environment in which they were derived. Cells from three wells were grown until subconfluent and then trypsinized, diluted 1:10, replated, grown, and counted. Student's t tests ($n = 3$) were performed to assign significance. Clonal lines were generated from EBD culture LV by low-density plating in EGM2MV media on collagen I (LVEC) followed by cloning cylinder isolation and expansion to $>1 \times 10^6$ cells. Cloning efficiency was determined by low density plating of a total of 600 LVEC cells. Methylene Blue staining to identify colonies was performed 10 days after plating. PD levels were calculated as 3.32 ($\log \text{cells}_{\text{harvested}} - \log \text{cells}_{\text{plated}}$) and do not include cell division during the initial phase of culture derivation.

Gene Transfer into EBD Cell Cultures. Stable transfection of human EBD cultures was carried out by lipofection. Briefly, 1–5 μg of a construct containing the neomycin phosphotransferase gene flanked by the mouse phosphoglycerate kinase-1 was used to transfect $\approx 2 \times 10^5$ cells by using Lipofectamine plus lipid (LTI). Stably transfected cells were selected by growth on collagen I in EGM2MV media supplemented with 200–400 μg/ml G418 and isolated by using cloning cylinders.

Retroviral transduction of culture LVEC was carried out by using the MGIN vector and amphotropic viral envelope as reported (13). This vector uses the retroviral long terminal repeat to drive transcription of enhanced green fluorescence protein (EGFP) and neomycin phosphotransferase coding regions. Lentiviral transduction was carried out by using the EF.GFP vector in which the human elongation factor 1α promoter drives the transcription of EGFP in an HIV-1 based self-inactivating lentiviral backbone. EF.GFP virus was produced by cotransfection of 293T cells with a packaging plasmid expressing HIV-1 gag/pol, REV and TAT proteins, and a plasmid expressing the VSV-G envelope. Viral titers were determined by the number of GFP expressing 293T cells after infection. For the EBD cell transduction, 1×10^5 LVEC cells were infected with 1×10^6 transducing units of either retrovirus or lentivirus in the presence of 8 μg/ml polybrene overnight for two successive days. Transduced cells were analyzed for GFP expression by using fluorescence-activated cell sorting analysis 6 days after plating and proliferation in the absence of drug selection. Mock-infected cells were used to establish the level of background fluorescence. Retrovirally transduced LVEC cells were selected by growth in the presence of 400 μg/ml G418.

Immunocytochemistry and Telomerase Activity Assay. Approximately 1×10^5 cells were plated in each well of an 8-well glass bottom chamber slide. Cells were fixed in either 4% paraformaldehyde in PBS or a 1:1 mixture of methanol/acetone for 10 min as recommended by the antibody manufacturer. Cells were permeabilized in 0.1% Triton X-100, 1 × PBS for 10 min if required, then blocked in Powerblock (BioGenex), 5% FBS, or 1–5% goat serum supplemented with 0.5% BSA for 10–60 min as recommended by the antibody manufacturer. Primary antibodies and dilutions were as follows: neurofilament 68 kDa (Roche, 1:4), neuron-specific enolase (PharMingen, 1:100), tau (PharMingen, 5 μg/ml), vimentin (Roche, 1:10), human nestin (gift from Ron McKay, National Institutes of Health, 1:250), galactocerebroside (Sigma, 1:500), 2′,3′-cyclic nucleotide 3′-phosphodiesterase (Sigma, 1:500), O4 (Roche, 10 μg/ml), and SMI32 (Sternberger monoclonal, 1:5000). Antibodies reactive to the astrocyte marker glial fibrillary acidic protein (GFAP) and neuronal marker β tubulin type III were not included, as conditions for their specific reactivity could not be established. Detection was carried out by secondary antibodies conjugated to biotin, streptavidin-conjugated horseradish peroxidase, and 3-amino-9-ethylcarbazole chromagen (BioGenex). Telomerase assays were performed by using a telomeric repeat amplification protocol followed by ELISA detection of amplified products (TeloTAGGG PCR ELISA PLUS, Roche).

mRNA Expression Profiles. RNA was prepared from cells growing on 60-mm tissue culture plates by using the Qiagen miniprep kit. RNA preparations were digested with RNase-free DNase (Roche) for 30 min at 37°C, and then the digest was inactivated at 75°C for 5 min. Synthesis of cDNA was performed on 5 μg of RNA by using oligo (dT) primers and a standard Moloney murine leukemia virus (LTI) reaction carried out at 42°C. Thirty cycles of PCR were carried out in the presence of 1.5 mM $MgCl_2$ with an annealing temperature of 55°C and incubation times of 30 s. PCR reactions were resolved on a 1.8% agarose gel. The efficacy of all PCRs was established by using appropriate commercially available human tissue RNA (CLONTECH). Some gels were subject to Southern blot analysis by using oligonucleotide probes end-labeled with [^{32}P]ATP, hybridized in 6 × SSC/5 × Denhardt's solution/0.1% SDS/0.05% sodium pyrophosphate/100 μg/ml sheared and denatured salmon sperm DNA at 45°C. cDNA synthesis and genomic DNA contamination were monitored by primers specific to human phosphoglycerate kinase-1, which give products of ≈250 bp and ≈500 bp when amplifying cDNA and genomic DNA, respectively. Ethidium bromide fluorescence of agarose gel resolved PCR amplimers, and immunocytochemical reactivities were subjectively assigned to one of four intensity categories: very strong, strong, detected, and not detected. PCR primer and probe sequences appear in Table 1, which is published as supplemental data on the PNAS web site, www.pnas.org.

Tumor Formation. Three female 6-week-old SCID-NOD mice were injected with 3×10^6 EBD cells (LVEC) or 2.5×10^5 to 1×10^6 mouse ES cells (ES D3) in the left and right calf muscles, respectively. After 1 month, animals were killed and visually examined for tumors. Injected calf muscles were dissected intact, fixed in 4% phosphate-buffered paraformaldehyde overnight, then processed and imbedded in paraffin. Sections stained with hematoxylin and eosin were examined.

Results

Human pluripotent stem cell cultures derived from primordial germ cells were isolated and cultured as described (12). During routine growth, 1–5% of the multicellular EG colonies formed large fluid-filled cystic EBs that were loosely attached to a remaining EG colony or to the fibroblast feeder layer. Four genetically distinct EG cultures (LV, SL, LU2, and SD) were selected to represent the range of developmental stages at which human EG cultures can be initiated (5–11 weeks postfertilization). Karyotypically, two cultures were 46, XX (LV, SD), and two were 46, XY (SL, LU2). Approximately 10 EBs were removed from each EG culture and were dissociated in a mixture of collagenase I and dispase I (Roche). EB constituent cells were then replated in three (LV) or six (SL, LU2, SD) growth media and biomatrix combinations in an effort to identify environments that promoted vigorous cell proliferation with the possibility of differential enrichment of outgrowth populations.

Two growth media were selected to investigate the effects of serum and specific mitogens on the proliferation of these human cells. RPMI 1640 supplemented with 15% FCS is a simple base media that relies on serum to support cell proliferation. EGM2MV(Clonetics) has a reduced serum content (5%) and contains basic fibroblast growth factor, epidermal growth factor, vascular epidermal growth factor, and insulin-like growth factor I. Three cell attachment surfaces were used: tissue culture-

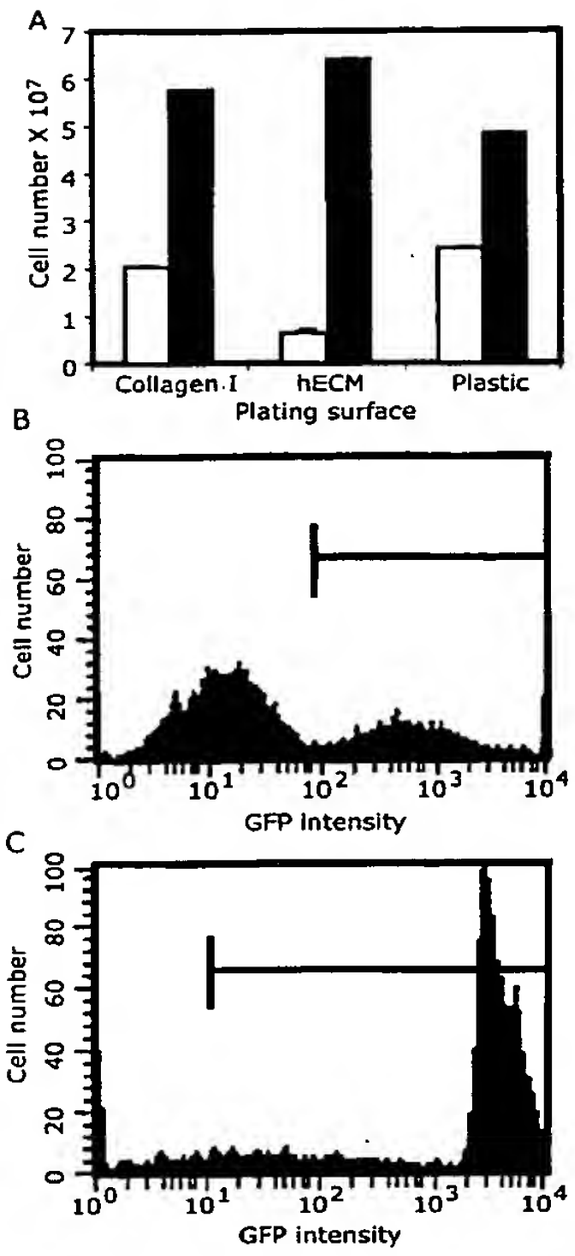

Fig. 1. EBD cell proliferation rate and flow cytometric (fluorescence-activated cell sorting) analyses. (*A*) 1×10^4 cells from EBD culture SL grown 6 days in RPMI/15% serum (unshaded bars) or EGM2MV media (shaded bars) on three plating surfaces then trypsinized and counted. Fluorescence-activated cell sorting analyses of retroviral (*B*) and lentiviral (*C*) transduction efficiency and EGFP expression level. Bars indicate levels above background.

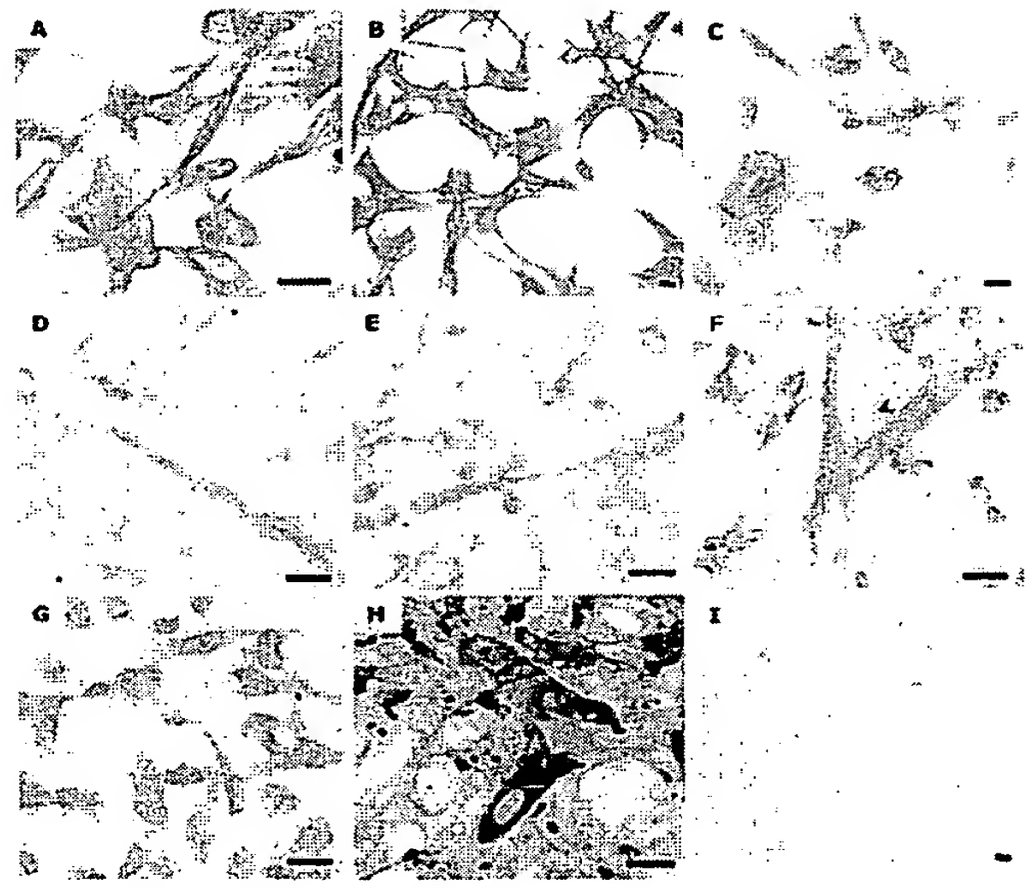

Fig. 2. Immunocytochemical analysis of EBD culture LVEC. Antibody epitopes are as follows: (*A*) nestin, (*B*) vimentin, (*C*) neurofilament light isoform, (*D*) tau, (*E*) neuron-specific enolase, (*F*) neurofilament heavy isoform, (*G*) CNPase, (*H*) galactocerebroside, and (*I*) O4 antigen, no immunoreactivity. (Bars represent 10 μm)

treated plastic, bovine collagen I, and human extracellular matrix extract. All six growth environments supported cell proliferation, and the resultant cells were termed EBD cell cultures. Cell proliferation studies carried out on several genetically distinct EBD cultures indicated that EGM2MV medium was superior to RPMI 1640 medium ($P < 0.001$) and that extracellular matrix and collagen I were superior to tissue culture plastic ($P < 0.001$). The results from a proliferation study on culture SD is shown in Fig. 1*A*. Karyotypic analysis performed on each culture at approximately 20 PD indicated that the cells had a normal diploid chromosomal complement.

The ability of EBD cells to be stably transfected in the EGM2MV/collagen I environment was examined by lipofection of a neomycin resistance gene driven by the mouse phosphoglucokinase I promoter. Stable integration efficiencies of $\approx 1 \times 10^{-5}$ were routinely obtained, and neomycin-resistant clonal lines could be expanded to $>1 \times 10^6$ cells. In an effort to improve integration efficiency, retroviral and lentiviral transduction were investigated. When cultures of LVEC were infected with equal titers of either retrovirus or lentivirus carrying EGFP expression vectors, the efficiencies were ≈30% and ≈98%, respectively, and remained constant for >2 weeks (Fig. 1 *B* and *C*). The retrovirally transduced LVEC culture was essentially 100% EGFP positive after 2 weeks of drug selection and has remained so for >30 PD.

In an effort to classify EBD cells by their expression characteristics, we initially chose to look for the presence of neural progenitor and neuronal and glial markers. Neural progenitors are capable of generating both neurons and glia and are known to express the intermediate filament proteins nestin (14) and vimentin (15–17). Additionally, we used several neuronal and glial markers in our survey, including neuronal markers neurofilament light isoform, tau, neurofilament heavy isoform (SMI32), and neuron-specific enolase; and glial markers 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), galactocerebroside, and O4 antigen.

As evident in Fig. 2, cells of the EBD culture LVEC were strongly immunoreactive to the nestin- and vimentin-specific antibodies (>95% cells positive). Cells were less strongly and/or less consistently immunoreactive (10–50% cells positive) to neurofilament light isoform-, tau-, neuron-specific enolase-, SMI32-, CNPase-, and galactocerebroside-specific antibodies. No cells immunoreactive for the O4 antigen were detected.

To confirm some of the antibody staining results, and to expand the range of markers examined, a 24-gene reverse transcriptase-PCR expression profile was carried out on the LVEC culture and other EBD cultures. Markers were chosen to indicate expression profiles of four cell lineages known to be present in human EBs: neural, muscle, vascular/hematopoietic, and endoderm (12). In this study, vascular and hematopoietic cells were grouped because they share the markers used. Additionally, the nine antibodies used in Fig. 2 were used, confirming the expression status of four PCR reactions, and extending the analysis to antigens not readily amenable to PCR. Expression of markers not verified by antibody staining was confirmed by Southern blot hybridization of PCR products to specific internal oligonucleotide probes (data not shown). When possible, several markers of a lineage or cell type were used. As seen in Fig. 3, the neural lineage markers were the most strongly and consistently expressed. Neural progenitor markers nestin and vimentin and astrocyte marker glial fibrillary acidic protein (GFAP) were expressed in all cultures. The neuronal markers neurofilament light isoform, microtubule associated protein 2C, tau, nonphosphorylated neurofilament heavy isoform (SMI32), neuron-specific enolase, and tyrosine hydroxylase were weakly expressed in many of the cultures, with occasionally stronger expression of

stem cells (33). In this model, multilineage gene expression by precursor or progenitor cells defines a ground state from which cell-extrinsic and cell-intrinsic signals work to continuously define a differentiated expression pattern and phenotype (34). In this regard, it would be useful to similarly assay the gene expression profiles of other stem, precursor, or progenitor cell populations.

The human cells described here have several properties that suggest their usefulness as models of human cell differentiation and in transplantation therapies. These include robust and long-term proliferation with a normal karyotype; ability to be cryopreserved, cloned, and genetically manipulated; and a developmentally broad multilineage expression profile. *In vitro* and *in vivo* animal model experiments are currently underway to evaluate the efficacy of EBD cells, with or without further differentiation, in the treatment of Parkinson's disease, amyotrophic lateral sclerosis (ALS), stroke, spinal cord injury, and diabetes. For PSCs to be of practical use, methods to generate large numbers of homogeneous cell types must be developed.

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